PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: JANET E. REED
SAUL EWING REMICK & SAUL LLP
CENTRE SQUARE WEST
1500 MARKET STREET, 38TH FLOOR
PHILADELPHIA PA 19102-2186

PCT

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of Mailing *(day/month/year)*	17 JUL 2001
Applicant's or agent's file reference RUTC 99-0034 13216.00024	IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority Date *(day/month/year)*
PCT/US00/11893	02 MAY 2000	04 MAY 1999

Applicant
RUTGERS, THE STATE UNIVERSITY OF NEW JERSEY

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer MANJUNATH RAO
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/IPEA/416 (July 1992)*

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference RUTC 99-0034	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US00/11893	International filing date *(day/month/year)* 02 MAY 2000	Priority date *(day/month/year)* 04 MAY 1999
International Patent Classification (IPC) or national classification and IPC IPC(7): C07K 1/00, 14/00, 17/00 and US Cl.: 530/350		
Applicant RUTGERS, THE STATE UNIVERSITY OF NEW JERSEY		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

   I [X] Basis of the report

   II [ ] Priority

   III [ ] Non-establishment of report with regard to novelty, inventive step or industrial applicability

   IV [ ] Lack of unity of invention

   V [X] Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

   VI [ ] Certain documents cited

   VII [ ] Certain defects in the international application

   VIII [X] Certain observations on the international application

Date of submission of the demand	Date of completion of this report
30 NOVEMBER 2000	21 JUNE 2001
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MANJUNATH RAO Telephone No. (703) 308-0196

Form PCT/IPEA/409 (cover sheet) (July 1998)*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/11893

I. Basis of the report

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed

[X] the description:
pages 1-21 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the claims:
pages 22-25 , as originally filed
pages NONE , as amended (together with any statement) under Article 19
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the drawings:
pages 1-6 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the sequence listing part of the description:
pages 1-6 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

[X] contained in the international application in printed form.

[X] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. [X] The amendments have resulted in the cancellation of:

[X] the description, pages NONE

[X] the claims, Nos. NONE

[X] the drawings, sheets/~~fig~~ NONE

5. [ ] This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

***Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

Intern[redacted] application No.
PCT/US00/11893

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. **statement**

Novelty (N)	Claims	1-20	YES
	Claims	NONE	NO
Inventive Step (IS)	Claims	1-20	YES
	Claims	NONE	NO
Industrial Applicability (IA)	Claims	1-20	YES
	Claims	NONE	NO

2. **citations and explanations (Rule 70.7)**

Claims 1-20 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest a chimeric protein with a repressor domain which represses the activity of the reporter domain fused thereto and a reporter domain having a detectable biological activity linked through a protease cleavage domain and the method s and kits based on such a system. The closest prior art is that of Xu et al. (Nucleic Acids Res., 1998, Vol. 26, No. 8:2034-2035) which teaches a similar system using fluorescence proteins.

---------- NEW CITATIONS ----------

NONE

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Claims 1, 10, 18 and 19 are objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 6 because claims 1, 10, 18-19 are indefinite for the following reason(s): Claims 1, 10, 18-19 recite the phrase "biologically active" or biologically active" for which no definition is provided by the applicants rendering the claims unclear.

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: JANET E. REED
SAUL EWING REMICK & SAUL LLP
CENTRE SQUARE WEST
1500 MARKET STREET, 38TH FLOOR
PHILADELPHIA PA 19102-2186

RECEIVED
IP GROUP
MAR 0 6 2001
SAUL EWING REMICK & SAUL LLP

JL 3/7/01

PCT

WRITTEN OPINION

(PCT Rule 66)

Due: 5/2/01

Date of Mailing *(day/month/year)* 02 MAR 2001	
Applicant's or agent's file reference RUTC 99-0034 13216.00024	REPLY DUE within **TWO** months from the above date of mailing
International application No. PCT/US00/11893	International filing date *(day/month/year)* 02 MAY 2000
Priority date *(day/month/year)* 04 MAY 1999	
International Patent Classification (IPC) or both national classification and IPC IPC(7): C07K 1/00, 14/00, 17/00 and US Cl.: 530/350	
Applicant RUTGERS, THE STATE UNIVERSITY OF NEW JERSEY	

1. This written opinion is the first (first, etc.) drawn by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

I	[X]	Basis of the opinion
II	[ ]	Priority
III	[ ]	Non-establishment of opinion with regard to novelty, inventive step or industrial applicability
IV	[ ]	Lack of unity of invention
V	[X]	Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	[ ]	Certain documents cited
VII	[ ]	Certain defects in the international application
VIII	[X]	Certain observations on the international application

3. The applicant is hereby invited to reply to this opinion.

When? See the time limit indicated above. ~~The applicant may, before the expiration of that time limit, request this Authority to grant an extension., see Rule 66.2(d).~~

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 *bis*.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 04 SEPTEMBER 2001

Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer MANJUNATH RAO
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Joyce Bridges for

Form PCT/IPEA/408 (cover sheet) (July 1998)*

WRITTEN OPINION

International application No.
PCT/US00/11893

I. Basis of the opinion

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed

[X] the description:
pages 1-21, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of

[X] the claims:
pages 22-25, as originally filed
pages NONE, as amended (together with any statement) under Article 19
pages NONE, filed with the demand
pages NONE, filed with the letter of

[X] the drawings:
pages 1-6, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of

[X] the sequence listing part of the description:
pages 1-6, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

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[X] contained in the international application in printed form.

[X] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. [X] The amendments have resulted in the cancellation of:

[X] the description, pages NONE

[X] the claims, Nos. NONE

[X] the drawings, sheets/~~fig~~ NONE

5. [ ] This opinion has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".*

WRITTEN OPINION

International application No.
PCT/US00/11893

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	1-20	YES
	Claims	NONE	NO
Inventive Step (IS)	Claims	1-20	YES
	Claims	NONE	NO
Industrial Applicability (IA)	Claims	1-20	YES
	Claims	NONE	NO

2. citations and explanations

Claims 1-20 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest a chimeric protein with a repressor domain which represses the activity of the reporter domain fused thereto and a reporter domain having a detectable biological activity linked through a protease cleavage domain and the method s and kits based on such a system. The closest prior art is that of Xu et al. (Nucleic Acids Res., 1998, Vol. 26, No. 8:2034-2035) which teaches a similar system using fluorescence proteins.

NEW CITATIONS

NONE

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Claims 1, 10, 18 and 19 are objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 6 because claims 1, 10, 18-19 are indefinite for the following reason(s): Claims 1, 10, 18-19 recite the phrase "biologically active" or biologically active" for which no definition is provided by the applicants rendering the claims unclear.

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

TIME LIMIT:

The time limit set for response to a Written Opinion may not be extended. 37 CFR 1.484(d). Any response received after the expiration of the time limit set in the Written Opinion will not be considered in preparing the International Preliminary Examination Report.

PA NT COOPERATION TREAT

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 21 March 2001 (21.03.01)	
International application No. PCT/US00/11893	**Applicant's or agent's file reference** RUTC 99-0034
International filing date (day/month/year) 02 May 2000 (02.05.00)	**Priority date** (day/month/year) 04 May 1999 (04.05.99)
Applicant LAM, Eric et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

30 November 2000 (30.11.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Henrik Nyberg
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PA NT COOPERATION TREAT

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING
AMENDMENTS OF THE CLAIMS

(PCT Rule 62 and
Administrative Instructions, Section 417)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as International Preliminary Examining Authority

Date of mailing (day/month/year)	
21 March 2001 (21.03.01)	
International application No.	International filing date (day/month/year)
PCT/US00/11893	02 May 2000 (02.05.00)

Applicant

RUTGERS, THE STATE UNIVERSITY OF NEW JERSEY et al

The International Bureau hereby informs the International Preliminary Examining Authority that no amendments under Article 19 have been received by the International Bureau (Administrative Instructions, Section 417).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Henrik Nyberg
Facsimile No. (41-22) 740.14.35	Telephone No. (41-22) 338.83.38

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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COMPOSITIONS AND METHODS FOR DETECTION OF ACTIVE PROTEASES

This application claims priority to U.S. Provisional Application No. 60/132,358, filed May 4, 1999, the entirety of which is incorporated by reference herein.

FIELD OF THE INVENTION

This invention relates to the field of detection and measurement of biological molecules. In particular, the invention provides a novel assay system for detecting the presence or amount of selected active proteases in biological samples.

BACKGROUND OF THE INVENTION

Various scientific articles are referred to in parentheses throughout the specification, and complete citations are listed at the end of the specification. These articles are incorporated by reference herein to describe the state of the art to which this invention pertains.

Proteases are ubiquitous enzymes that play important roles in the control of cellular processes. In eukaryotes, proteases play key roles in orchestrating the progression of the cell cycle as well as in the decision process for activating programmed cell death. For example, it has become clear in the past 10 years that a large number of distinct but related cysteine proteases, called caspases, are involved in cell death activation in animals (Cryns and Yuan, 1998). From gene knock-out studies in mice, it is quite clear that different caspases play distinct roles in the cell death control of various tissues. In addition, although aspartate is the invariant residue at the P1 position of their target sites, animal caspases can be distinguished from each other by their preference of distinct substrate peptide sequences (Talanian et al. 1997). The ability to monitor the presence of different caspases in vivo should greatly facilitate our understanding of how this family of important protease may be controlled at the level of their enzymatic activity.

Many other proteases also recognize distinct targets for cleavage of proteins. These include cathepsin G, papain and thrombin, to name a few.

One *in vivo* approach for monitoring protease activity is the technology of Fluorescence Resonance Energy Transfer (FRET). In the approach described by Heim and Tsien (1995), two fluorescent proteins, Green Fluorescent Protein (GFP) and Blue Fluorescent Protein (BFP) are linked by a 25 amino acid linker with a trypsin cleavage site. FRET from BFP to GFP can be demonstrated with the fusion protein and is lost upon trypsin addition. The lost of FRET is measured as a decrease of green fluorescence with the concomitant increase in blue fluorescence. Although this technique can in theory be used to detect the presence of proteases *in vivo*, the approach is likely to suffer from lack of sensitivity. Thus, if the activity of a particular protease in a cell is low or transient, the lost of FRET in a small percentage of the expressed GFP-BFP fusion proteins may be difficult to detect. Furthermore, the assay for FRET requires sophisticated and expensive equipment and background fluorescence of particular biological organisms (such as plants) may limit the application of this technology.

From the foregoing discussion, it can be seen that there is a need for economical and sensitive screening strategies for measuring the activities of selected proteases *in vivo* or in cell-free extracts. Such strategies would advance the field in several respects, which include facilitating the discovery of novel proteases and drugs that can modulate specific protease activities in different cellular contexts.

SUMMARY OF THE INVENTION

The present invention provides a novel assay system for measuring the activity of selected proteases in a variety of biological systems. The compositions and methods of the invention can be used *in vivo* or in cell-free assays to measure the activity of one or more selected proteases.

According to one aspect of the invention, a chimeric protein for detecting the presence or activity of a pre-determined protease is provided. The chimeric protein contains (a) a repressor domain which represses activity of a normally biologically active protein fused thereto; (b) a reporter domain comprising a

protein having a detectable biological activity when not fused to the repressor domain; and (c) a protease cleavage domain linking the repressor domain to the reporter domain, the protease cleavage domain comprising a structure that is cleaved by activity of the pre-determined protease. In a preferred embodiment, the repressor domain is a hormone binding domain of a steroid hormone receptor, the reporter domain is β-glucuronidase and the protease cleavage domain is a cleavage site for a caspase.

In another embodiment, the chimeric protease detector protein comprises at least one repressor domain and a plurality of reporter domains, each linked to the repressor domain(s) by a protease cleavage site. Using a multiplicity of reporters and cleavage sites, this protease detector protein can be used to detect more than one selected protease.

According to another aspect of the invention, a method is provided for determining the presence or activity of a pre-determined protease in a biological sample, which utilizes the chimeric protease detector protein described herein. The method comprises adding the protease detector protein to the biological sample suspected of containing the pre-determined protease and measuring the detectable biological activity, if any, of the reporter domain. The occurrence and amount of the detectable biological activity is proportional to the occurrence and amount of the pre-determined protease in the biological sample.

The aforementioned method may be used in a biological sample comprises a biological fluid, tissue or cell extract by providing the protease detector protein as an isolated protein. Alternatively, the method can be used in a biological sample comprising intact cells in which the pre-determined protease, if present, is contained. In this instance, the protease detector protein is provided by introducing into the cells an expressible DNA construct that encodes the protein, under conditions whereby the protein is expressed. The DNA construct may be stably or transiently introduced into the cells.

According to another aspect of the invention, the above-described methods can be adapted for determining the presence or amount of a plurality of pre-determined proteases. This is accomplished by adding a plurality of protease detector

proteins to the sample, each having a protease cleavage domain specifically cleaved by one of the pre-determined proteases, and each having a differentially detectable reporter domain. Alternatively, one or more modified protease detector proteins can be used, each comprising a repressor domain linked to two different protease cleavage domain, each protease cleavage domain being linked to a differentially detectable reporter domain.

In another aspect of the invention, the foregoing methods can be used in a screening assay to determine if a test compound affects the amount or activity of a pre-determined protease. In a typical screening assay, the selected protease and protease detector are combined in the presence or absence of the test compound, and the amount of detectable reporter is measured. An increase or decrease in detectable reporter production in the presence of the test compound is indicative of the ability of that compound to affect the amount or activity of the protease.

According to another aspect of the invention, test kits are provided for performing one or more of the assays described herein. The kits contain one or more of the protease detector proteins and, optionally, additional reagents and instructions for performing the assays.

Other features and advantages of the present invention will be better understood by reference to the drawings, detailed description and examples that follow.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Design of an exemplary protease detector of the present invention. The hormone binding domain (HBD) of the glucocorticoid receptor is fused to the β-glucuronidase (GUS) reporter gene. Between the proteins is introduced a protease recognition cleavage site. The GUS protein is inactive while attached to the HBD because it is unable to dimerize. The protease detector comprises a caspase-1 cleavage site. The control comprises a modified caspase-1 cleavage site, which is not recognized for cleavage by caspase-1. Upon addition of caspase-1 to the system, cleavage and release of GUS is observed in the protease detector, but not in the control.

Figure 2. Schematic diagram showing the method for the construction of the fusion proteins described in Example 1. Fig. 2A: shows step1, introduction of the caspase target cleavage site by polymerase chain reaction (PCR). Fig. 2B: shows step 2, creating an intermediate chimeric clone, 3'GUS-YVADG-HBD. Fig. 2C: shows step 3, 5' end GUS gene reconstruction.

Figure 3. Autoradiogram of SDS-PAGE gel demonstrating that the linker site between GUS and GR-HBD can be specifically recognized and cleaved by purified caspase-1. Lefthand 3 lanes show protease detector GUS-YVAD-HBD, in the presence or absence of caspase-1 and/or a peptide inhibitor of caspase-1, AcYVAD-CMK; center 3 lanes show control construct GUS-YVAA-HBD that is not recognized by caspase-1, in the presence or absence of caspase-1 and/or a peptide inhibitor of caspase-1; righthand 3 lanes show GUS alone, in the presence or absence of caspase-1 and/or a peptide inhibitor of caspase-1.

Figure 4. Graphs showing GUS activity from *in vitro* translated samples using various constructs. Fig. 4A shows results of an experiment in which reticulocyte lysate alone, or expressing the caspase-1 detector construct in the presence of caspase, were examined for GUS activity. Fig. 4B shows results of a second experiment that tested GUS activity in *in vitro* translations using a variety of control and test combinations. Legend: Ret = reticulocyte lysate; casp1 = caspase-1; Ac-YVAD-CMK = peptide inhibitor of caspase-1; TGUS-YVAD-HBD = the caspase-1 protease detector; C = conrol (no caspase-1); TGUS-YVAA-HBD = the caspase-1 protease detector control construct.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions:

Various terms relating to the present invention are used hereinabove and also throughout the specifications and claims.

A "coding sequence" or "coding region" refers to a nucleic acid molecule having sequence information necessary to produce a gene product, when the sequence is expressed.

The term "operably linked" or "operably inserted" means that the

regulatory sequences necessary for expression of the coding sequence are placed in a nucleic acid molecule in the appropriate positions relative to the coding sequence so as to enable expression of the coding sequence. This same definition is sometimes applied to the arrangement other transcription control elements (e.g. enhancers) in an expression vector.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

The terms "promoter", "promoter region" or "promoter sequence" refer generally to transcriptional regulatory regions of a gene, which may be found at the 5' or 3' side of the coding region, or within the coding region, or within introns. Typically, a promoter is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. The typical 5' promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence is a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

A "vector" is a replicon, such as plasmid, phage, cosmid, or virus to which another nucleic acid segment may be operably inserted so as to bring about the replication or expression of the segment.

The term "nucleic acid construct" or "DNA construct" is sometimes used to refer to a coding sequence or sequences operably linked to appropriate regulatory sequences and inserted into a vector for transforming a cell. This term may be used interchangeably with the term "transforming DNA" or "transgene". Such a nucleic acid construct may contain a coding sequence for a gene product of interest, along with a selectable marker gene and/or a reporter gene.

The term "selectable marker gene" refers to a gene encoding a product that, when expressed, confers a selectable phenotype such as antibiotic resistance on a

transformed cell.

The term "reporter gene" refers to a gene that encodes a product which is easily detectable by standard methods, either directly or indirectly.

A "heterologous" region of a nucleic acid construct is an identifiable segment (or segments) of the nucleic acid molecule within a larger molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. In another example, coding sequence is a construct where the coding sequence itself is not found in nature (e.g., a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

A cell has been "transformed" or "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA (transgene) may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations. If germline cells are stably transformed, the transformation may be passed from one generation of animals arising from the germline cells, to the next generation. In this instance, the transgene is referred to as being inheritable.

Other definitions are found in the description set forth below.

II. Description:

The present invention provides a sensitive and versatile detection system for proteases. The invention arises from the inventors' insight that the ligand binding domain of a certain class of cellular receptor molecules can be used to mask a well-characterized enzyme with activity that can normally be easily monitored. When a protease target site is positioned between these two domains of the chimera and the corresponding protease is added, the cleavage of the target sequence results in the appearance of the previously masked enzyme activity. The "gain-of- function" nature of this assay system provides high sensitivity and versatility in the monitoring of protease activities *in vitro* as well as *in vivo*.

The inventors have tested this novel strategy for the detection and monitoring of protease activities for in vitro and in vivo studies. As described in Example 1, the hormone binding domain (HBD) of the rat glucocorticoid receptor (GR) was fused to the bacterial enzyme β-glucuronidase (GUS) with a peptide sequence (YVADG) for caspase-1 cleavage inserted as a linker. When translated *in vitro* with rabbit recticulocyte lysates or expressed in transgenic plants (tobacco and Arabidopsis), the fusion of GUS to the HBD of GR resulted in complete inactivation of its enzymatic activity. *In vitro*, the inventors demonstrated that cleavage of GUS-GR by addition of caspase-1 leads to the release of the GUS protein from the GR domain with concomitant appearance of GUS activity. Introduction of a single point mutation in the P1 position of the caspase cleavage site (YVAAG) abolished cleavage of the fusion protein by added caspase-1 and loss of GUS activation. These results illustrate the principle of the invention by demonstrating that HBD domains of steroid receptors can be used to inactivate enzyme activities of attached protein partners. The fusion junction is accessible to proteolytic enzymes, and cleavage by the appropriate protease leads to the separation of the HBD domain from the enzyme partner and results in the unmasking of the latent enzymatic activity.

The following description sets forth the general procedures involved in practicing the present invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. Unless otherwise specified, general cloning procedures, such as those set forth in

Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory (1989) (hereinafter "Sambrook et al.") or Ausubel et al. (eds) Current Protocols in Molecular Biology, John Wiley & Sons (2000) (hereinafter "Ausubel et al.") are used.

The detection system of the invention utilizes a three-part chimeric protein, referred to herein as a "protease detector". The protease detector is composed of (1) a "repressor domain"; (2) a "reporter domain" comprising an enzyme that, when liberated from the repressor domain, has activity which is easily detectable; and (3) a protease cleavage domain that joins the repressor domain to the reporter domain. The protease detector is introduced into a test sample containing, or suspected of containing, a protease that specifically recognizes the protease cleavage domain. If present, the protease cleaves the chimeric detector at the cleavage domain, thereby liberating the reporter domain and allowing it to become active. Enzymatic activity of the reporter domain is detected, and the presence or amount of that activity is correlated to the presence or amount of the protease in the test sample.

The repressor domain can be any protein domain that represses the activity of a reporter enzyme to which it is linked via the protease cleavage domain. In preferred embodiments, repressor domains are taken from cellular receptors whose activity in cells is repressed until activated by binding of its cognate ligand. In accordance with the present invention, however, it has been found that, these repressor domains can act as ligand-independent repressors of activity of enzymes linked to them via a protease cleavage domain. Thus, the present invention differs in its fundamental nature from assays using ligand-activated receptors such as HBD, in that this system does not need to be activated by ligand binding to the receptor. Activation takes place only after protease cleavage.

The steroid hormone receptors are examples of cellular receptors whose binding domains provide particularly suitable repressor domains for use in the protease detector proteins of the invention. The steroid hormone receptors are members of a large family of important transcriptional regulators in animal systems. These proteins functions to transduce signals from steroid hormones to control cellular processes via the control of gene expression in the nucleus. The hormone binding domain (HBD) of the steroid receptor acts as a regulatory domain to control

the function of the transcription factor domain (TFD). In the absence of the hormone, the HBD represses the activity of the TFD via the interaction with the heat shock protein HSF90. When hormone is present and binds to the HBD, the receptor is released from the HSP90 complex and the TFD is allowed to form a functional dimer and translocate into the nucleus to activate gene expression. It has been found that the HBD of steroid receptors such as the glucocorticoid receptor (GR) can function as an autonomous steroid regulatory domain hormone-binding domains (HBD) and can be used in various systems to confer steroid-dependent enzymatic activities (reviewed in Mattioni et al. 1994). In addition to animal cells, the HBD of GR has also been shown to confer dexamethasone-dependent transcription activities in higher plants (Simon et al. 1996; Aoyama and Chua 1997).

Another family of cellular receptors that contain suitable repressor domains is the bHLH/PAS superfamily of transcription regulators, exemplified by the aryl hydrocarbon receptor (AHR) and the hypoxia inducible factors (HIFα and HIF3α). The distinguishing characteristic of these proteins is a 200-300 stretch of amino acid sequence similarity known as a PAS (PER/ARNT/SIM) domain. The helix-loop-helix domain serves as a dimerization surface for AHR and ARNT and also positions the basic α-helix within the major groove of B-DNA to enable specific interactions with target enhancer elements. The PAS domain, a region of ~250 amino acids, functions as a dimerization surface, harbors a repressor region, and also contains regions required for binding agonist and forming interactions with Hsp90. The AHR resides primarily in the cytosol, where it is associated in an inactive form with a dimer of the molecular chaperone, Hsp90. Upon binding an agonist, the AHR dissociates from Hsp90, translocates to the nucleus and dimerizes with a structurally related protein, ARNT. This complex interacts with enhancer elements upstream of target promoters and up-regulates the transcription of a variety of xenobiotic metabolizing enzymes (e.g., the Cyt P450 encoded by CYP1A1). The AHR and ARNT are both members of the basic helix-loop-helix-PAS superfamily.

The reporter domain can be any domain that, when attached to the repressor domain through the protease cleavage domain, is substantially undetectable, but when detached from the repressor domain by protease cleavage, becomes

detectable. Detectability can be by any means, but preferably relates to biological activity that is regained upon liberation of the reporter domain from the repressor domain. Examples of reporter domains suitable for use in the present invention include, but are not limited to, β-glucuronidase (GUS), β-galactosidase, chloramphenicol acetyl transferase (CAT), various transcription factors, alcohol dehydrogenase and luciferase.

The protease cleavage domain links the repressor domain to the reporter domain. This domain comprises a peptide sequence specifically recognized and cleaved by the protease whose activity is being assayed. A variety of proteases recognize specific cleavage sites in polypeptide sequences. Examples are set forth in the table below, which contains a list of characterized proteases and their specific substrates. Cleavage takes place between amino acid residue X and the P1 position for each of the target sites (X represents any amino acid) (Source: http//delphi. phys.univ-tours.fr/ Prolysis/sublist.html and the catalog from Calbiochem Co.).

Protease	**Target site**		
	3-letter code	**1-letter code**	**SEQ ID NO:**
Calpain	Val-Leu-Lys-X	VLKX	10
Cathepsin G	Ala-Val-Pro-Phe-X	AVPFX	11
Collagenase	Pro-Gln-Gly-Ile-Ala-Gly-Gln-X	PQGIAGQX	12
Elastase I	Ala-Ala-Pro-Val-X	AAPVX	13
Elastase II	Ala-Ala-Pro-Ala-X	AAPAX	14
Granzyme B	Ala-Ala-Asp-X	AADX	15
MMP-1	Pro-Gln-Gly-Ile-Ala-Gly-Gln-DArg-X	PGGIAGQrX	16
Kallikrein	Pro-Phe-Arg-X	PFRX	17
Papain	Gln-Val-Val-Ala-		

	Gly-Ala-X	QVVAGAX	18
Renin	Arg-Pro-Phe-His-Leu-		
	Leu-Val-Tyr-X	RPFHLLVYX	19
Thrombin	Val-Pro-Arg-X	VPRX	20

Caspases	Preferred target site	SEQ ID NO:
Caspase-1	YVADX	1
Caspase-2	VDVADX	2
Caspase-3	DEVDX	3
Caspase-4	LEVDX	4
Caspase-5	WEHDX	5
Caspase-6	VEIDX	6
Caspase-7	VDQVDX	7
Caspase-8	IETDX	8
Caspase-9	LEHDX	9

It may be determined in some cases that, due to the size of the protease, the size of the cleavage site, or the selection of repressor or reporter domains, the protease cleavage domain is not efficiently cleaved by the protease. In this situation, the difficulty may be resolved by extending the protease cleavage site on one or both ends with an additional length of peptide, sometimes referred to herein as a "spacer" or a "linker".

The chimeric constructs of the invention are composed of proteins or peptides linked together. This may be accomplished in one of several ways known in the art, as summarized below.

Peptides may be prepared by various synthetic methods of peptide synthesis via condensation of one or more amino acid residues, in accordance with conventional peptide synthesis methods. Preferably, peptides are synthesized according to standard solid-phase methodologies, such as may be performed on an Applied Biosystems Model 430A peptide synthesizer (Applied Biosystems, Foster

City, CA), according to manufacturer's instructions. Other methods of synthesizing peptides or peptidomimetics, either by solid phase methodologies or in liquid phase, are well known to those skilled in the art. When solid-phase synthesis is utilized, the C-terminal amino acid is linked to an insoluble resin support that can produce a detachable bond by reacting with a carboxyl group in a C-terminal amino acid. One preferred insoluble resin support is *p*-hydroxymethylphenoxymethyl polystyrene (HMP) resin. Other useful resins include, but are not limited to: phenylacetamidomethyl (PAM) resins for synthesis of some N-methyl-containing peptides (this resin is used with the Boc method of solid phase synthesis; and MBHA (p-methylbenzhydrylamine) resins for producing peptides having C-terminal amide groups.

During the course of peptide synthesis, branched chain amino and carboxyl groups may be protected/deprotected as needed, using commonly-known protecting groups. In a preferred embodiment, N^{α}-amino groups are protected with the base-labile 9-fluorenylmethyloxycarbonyl (Fmoc) group or *t*-butyloxycarbonyl (Boc groups). Side-chain functional groups consistent with Fmoc synthesis may be protected with the indicated protecting groups as follows: arginine (2,2,5,7,8-pentamethylchroman-6-sulfonyl); asparagine (O-t-butyl ester); cysteine glutamine and histidine (trityl); lysine (*t*-butyloxycarbonyl); serine and tyrosine (*t*-butyl). Modification utilizing alternative protecting groups for peptides and peptide derivatives will be apparent to those of skill in the art.

Full-length proteins or protein domains for use in the present invention may be prepared in a variety of ways, according to known methods. Proteins may be purified from appropriate sources, e.g., human or animal cultured cells or tissues, by various methods such as gel filtration, ion exchange chromatography, reverse-phase HPLC and immunoaffinity purification, among others. However, due to the often limited amount of a protein present in a sample at any given time, conventional purification techniques are not preferred in the present invention.

The availability of nucleic acids molecules encoding a protein enables production of the protein using *in vitro* expression methods known in the art. For example, a cDNA or gene may be cloned into an appropriate *in vitro* transcription

vector, such a pSP64 or pSP65 for *in vitro* transcription, followed by cell-free translation in a suitable cell-free translation system, such as wheat germ or rabbit reticulocytes. *In vitro* transcription and translation systems are commercially available, e.g., from Promega Biotech, Madison, Wisconsin or BRL, Rockville, Maryland. Example 1 describes the use of this methodology in constructing a protease detector comprising GUS, the HBD from GR and a caspase-1 cleavage site.

Alternatively, according to a preferred embodiment, a selected peptide or protein may be produced by expression in a suitable procaryotic or eucaryotic system. For example, a DNA molecule, encoding a peptide or protein component of the invention, or an entire chimeric protease detector molecule of the invention, may be inserted into a plasmid vector adapted for expression in a bacterial cell, such as *E. coli*, or into a baculovirus vector for expression in an insect cell. Such vectors comprise the regulatory elements necessary for expression of the DNA in the host cell, positioned in such a manner as to permit expression of the DNA in the host cell. Such regulatory elements required for expression include promoter sequences, transcription initiation sequences and, optionally, enhancer sequences.

A peptide or protein produced by gene expression in a recombinant procaryotic or eucaryotic system may be purified according to methods known in the art. In a preferred embodiment, a commercially available expression/secretion system can be used, whereby the recombinant protein is expressed and thereafter secreted from the host cell, so as to be readily purified from the surrounding medium. If expression/secretion vectors are not used, an alternative approach involves purifying the recombinant protein by affinity separation, such as by immunological interaction with antibodies that bind specifically to the recombinant protein. Such methods are commonly used for isolating peptides and proteins.

In an alternative embodiment, protein and/or peptide components of the invention are synthesized separately, then conjugated using standard methods known by those skilled in the art. For example, a synthetic peptide may be chemically coupled to a protein using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBF). This reagent cross-links amino- and carboxy-terminal thiol groups in the peptide with lysine side chains present in the protein. Alternatively, a synthetic peptide may be

coupled to a protein using glutaraldehyde, a common cross-linking agent. Another method for chemically coupling a peptide to a protein is through the use of carbodiimide and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide (EDC). Methods for joining two proteins together are also available.

The peptides or proteins of the invention, prepared by the aforementioned methods, may be analyzed according to standard procedures. For example, they may be subjected to amino acid sequence analysis, mass spectra analysis or amino acid compositional analysis according to known methods.

The chimeric protease detector proteins of the present invention comprise two domains linked together by a protease cleavage site with or without linkers. The organization of the respective domains can differ. For instance, if "R" represents the repressor domain, "D" represent the detectable reporter domain, and "C" represents the protease cleavage domain (with or without linkers), the protease detector proteins of the present invention may be organized as follows:

D - C - R;

R - C - D;

D_1 - C_1 - R - C_2 - D_2; wherein subscripted numbers indicated different cleavage sites or reporter domains. It should also be apparent that multiple repressor domains can be used in designing a complex protease detector protein.

The protease detector proteins of the present invention can be used singly or in combination to detect and quantitate activity of selected proteases *in vitro* and *in vivo*. The proteins can be used to assay various biological fluids, including tissue or cell extracts or environmental samples for activity of pre-determined proteases. Furthermore, *in vitro* detection of protease activation will enable the screening for drugs that affect cellular processes where proteases are activated either directly or upstream in a signal transduction pathway. The addition of appropriate substrates for the particular reporter enzyme in a microtiter plate will enable one to correlate protease activation with reporter enzyme activity. In the reverse approach, it can allow one to detect the presence of protease inhibitors or inhibitors of upstream components of the protease signaling pathway which normally leads to protease

activation, thus rendering no reporter enzyme activity under inducing conditions.

In addition to their applications for enzymatic assays *in vitro*, by using histochemical substrates such as X-Gluc for GUS, the protease detectors of the invention can be used to detect and quantitate cell-specific induction of protease activities in transformed organisms. In addition, through the selection and use of distinct enzymes that can work with fluorogenic or histochemical substrates that can produce products with different colors or chemical properties, the protease detectors of the invention can be used for multiplex analyses of different protease activities in the same cell.

It may also be desirable to design continuous monitoring systems for proteases in eukaryotes. For this purpose, an indirect activation/amplification system may be appropriate. In this approach, a well-defined heterologous transcription factor, such as the yeast transcription factor Gal4 may be fused with the repressor domain via the protease cleavage domain. The transcription factor remains inactive until cleavage from the repressor. As a reporter for the released transcription factor, a reporter gene (e.g., Green Fluorescent Protein or luciferin) expression cassette is placed under the control of a promoter consisting of the DNA responsive element to which the transcription factor binds (e.g., one or more Gal4 operator sequences). It is known that this type of synthetic promoter is stringently dependent on the presence of the transcription factor protein, with little background in either plant or animal cells. These constructs are thereafter introduced into cells of interest, where they are expressed. This strategy enables continuous monitoring of the level of a particular protease activity via detection of the reporter gene product in the cells. If differentiable detectable reporters are used(e.g., different spectral variants of GFP), the large numbers of well-characterized transcription regulators from bacteria and fungi should allow monitoring of the activities of multiple proteases simultaneously. This assay system can be employed using any of the numerous well-characterized transcription factor systems presently available in the art.

Test kits are also provided in accordance with the present invention, to facilitate the use of the protease detector in cell-free or cell-based assays such as those described above. In a preferred embodiment, the kit is a protease detection kit that

comprises a chimeric protease detector protein as describe above, along with instructions on how to use the protein to detect the presence or activity of a pre-determined protease and, optionally, further comprises at least one other reagent useful for conducting assays to detect the presence or activity of a protease. In a particularly preferred embodiment, the test kit is adapted for detection of a plurality of pre-determined proteases, and comprises two or more different protease detector proteins.

The invention provides another test kit useful for continuous monitoring of protease activity in a selected cell type. This kit provides a protease detector system having two constructs; one in which a transcription factor is linked to a repressor moiety via the protease cleavage site, and the other comprising a reporter gene under the control of a promoter and the DNA responsive element activated by binding of the transcription factor. This kit also may provide selected cell types for practicing the assay, along with various reagents for culturing the cells, introducing the constructs into the cells, and detecting expression of the reporter gene.

The following example is provided to describe the invention in greater detail. It is intended to illustrate, not to limit, the invention.

EXAMPLE 1

HBD/GUS Construct for Detecting Active Caspase

In this example, it is experimentally determined whether the HBD of GR and other steroid receptors could possibly act as general repressor domains that could be used to mask the enzyme activities of a protein fusion partner. The placement of a defined protease target site sequence between the two partners would then release the latent enzymatic activity from repression by the HBD and the associated HSP90.

A prerequisite for this strategy is the accessibility of the protease target site in the fusion protein and the ability of the released enzyme to regain its active state. Prior to the experimental results reported herein, it was entirely unclear whether the cleavage site would be available or, if available, if the released reporter enzyme

would be active. We proceeded to test this approach by the strategy outlined in Figure 1. Since Caspase-1 from animal systems have been well-characterized, we chose its target sequence YVADG as our first test case. The coding sequence for the bacterial enzyme β-glucuronidase (GUS) is fused to the HBD of GR with the peptide sequence YVADG as a linker between the two partners of the chimera. As a negative control for sequence specific cleavage by caspase-1, an almost identical fusion protein between GUS and the HBD is created with the linker YVAAG. The conversion of the aspartate at the P1 position in the linker is predicted to abolish cleavage by caspase-1. If our strategy is operating as designed, we would predict that the two fusion proteins will both be inactive upon their synthesis in the presence of HSP90. Upon addition of caspase-1, the cleavage of the YVADG sequence may lead to the appearance of GUS activity while the fusion with the YVAAG linker should be unaffected.

The method for the construction of the fusion proteins are described in Figure 2. In the first step, the caspase target sequence and its P1 variant are introduced to the 3' end of the GUS gene and the two ends of the GR-HBD coding sequence via PCR amplification using synthetic oligonucleotide primers. These clones allow one to create either N-terminal or C-terminal fusions between the two partners with the same protease target site as linker in both cases. For the present work, we only created and tested fusions with GUS as the N-terminal partner. In step II, the 3' portion of GUS with the appropriate protease site linker is fused with the HBD to generate a partial fusion. The full length GUS coding sequence is then reconstituted in Step III by subcloning into the appropriate sites of the vector pBI221. This strategy avoids the need to amplify the complete coding sequence for GUS each time a new linker sequence needs to be constructed.

Figure 3 presents the results that demonstrate the linker site between GUS and GR-HBD can be specifically recognized and cleaved by purified caspase-1. The two fusion constructs as well as GUS alone were inserted into a pET vector (Novagen) for T7 RNA polymerase dependent in vitro transcription/translation using a coupled rabbit recticulocyte lysate system (Promega). To visualize the translated proteins, [35S]methionine was incorporated into the newly synthesized products. Figure 3 shows that fusion proteins of about 100 kDa were produced with the two

constructs while the GUS alone construct produced a protein with an apparent mass of about 70 kDa. Addition of purified caspase-1 generated cleavage products of 70 kDa and about 30 kDa from the YVADG containing fusion but not the P1 linker variant. In fact, no obvious proteolysis was detected by caspase-1 was observed with either the P1 linker mutant fusion or the GUS alone control. As expected, the cleavage of the YVADG containing fusion protein by caspase-1 can be inhibited by addition of the caspase specific peptide inhibitor YVAD-cmk.

To assay for the activity of the fusion proteins, we carried out *in vitro* transcription/translation of the different constructs without radiolabelled methionine. The results are presented in Figure 4. In the absence of caspase-1, either fusion protein show essential no significant GUS activity above the low background present in reticulocyte lysates alone. In a separate experiment, we found that transcription/translation with the GUS alone control can produce high GUS activity with this assay system (data not shown). Thus, the absence of GUS activity with the fusion proteins is likely due to the inactivation of the GUS partner by its fusion to the GR-HBD domain and not due to the presence of inhibitory compounds in the lysate. Upon addition of caspase-1 to the YVADG containing fusion protein, a dramatic appearance of GUS is observed. This is in contrast to the YVAAG containing fusion protein, in which case no detectable increase in GUS activity is observed. The unmasking of the GUS activity in the fusion is due to the proteolytic cleavage by the added caspase-1 since inclusion of the caspase specific inhibitor YVAD-cmk abolished this process. These results demonstrate that the released GUS enzyme partner can function properly as a reporter of active caspase cleavage.

In this example, we documented the successful application of the steroid hormone receptor as a sensitive reporter system to detect active protease *in vitro*. Using GUS as a model reporter, we found that fusion of the GR-HBD can effectively mask the intrinsic enzyme activity of the GUS partner in the chimeric protein produced in the rabbit reticulocyte lysate system. Quantitative kinetic analysis of GUS enzyme activity demonstrated no detectable cleavage of the fluorogenic substrate 4-MUG by the fusions. The tightness of this repression by the HBD domain is likely linked to the fact that the GUS protein needs to dimerize in order to form the

active enzyme. Our observation thus indicate that there is a likely excess of HSP90 in the lysate that we used to produce the fusion protein *in vitro*. The effective sequestration of the translated GUS-HBD fusion protein by the presumed association with HSP90 then resulted in complete suppression of GUS enzymatic activity. These *in vitro* results are corroborated by our analyses with transgenic plants (specifically tobacco and *Arabidopsis*), in which the GUS-HBD fusions were expressed under a strong constitutive viral promoter. Although these plants were found by RNA gel blot analyses to express high levels of transcripts for the transgene, little GUS activity can be detected (data not shown). This result suggests that the GR-HBD domain can also effectively repress the GUS fusion partner when expressed in eukaryotic cells, identical to our observation *in vitro*.

Previous work with chimeric fusions of steroid receptor HBDs have documented that addition of the appropriate hormone can relieve the repressive function of this domain on the fusion partner. However, it was not clear that the linker region between the two partners in the chimera can in fact be accessible to externally added enzymes such as proteases. Our present work demonstrated that insertion of a protease site into this junction region is in fact quite readily cleaved in a specific manner by added protease. Furthermore, the separated reporter protein readily regained its activity. Addition of an inhibitor specific for the added protease inhibited cleavage of the fusion protein and abolished the activation of the latent enzyme activity. These results thus demonstrated that the HBD of steroid hormones can be linked to various reporter enzymes such as GUS to provide a variety of chimeric protease reporters.

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The present invention is not limited to the embodiments described and exemplified above, but is capable of variation and modification without departure from the scope of the appended claims.

We claim:

1. A chimeric protein for detecting the presence or activity of a pre-determined protease, which comprises:

a) a repressor domain which represses activity of a normally biologically active protein fused thereto;

b) a reporter domain comprising a protein having a detectable biological activity when not fused to the repressor domain; and

c) a protease cleavage domain linking the repressor domain to the reporter domain, the protease cleavage domain comprising a structure that is cleaved by activity of the pre-determined protease.

2. The chimeric protein of claim 1, wherein the repressor domain comprises a hormone binding domain of a steroid hormone receptor.

3. The chimeric protein of claim 1, wherein the reporter domain comprises β-glucuronidase.

4. The chimeric protein of claim 1, wherein the protease cleavage domain comprises a cleavage site for a caspase.

5. The chimeric protein of claim 1, which further comprises a spacer between the protease cleavage domain and one or both of the repressor domain and the reporter domain.

6. The chimeric protein of claim 1, which comprises at least one repressor domain and a plurality of reporter domains, each linked to the at least one repressor domain by a protease cleavage site.

7. The chimeric protein of claim 7, wherein the plurality of reporter domains are different from one another.

8. The chimeric protein of claim 7, wherein the protease cleavage sites are different from one another.

9. A chimeric protein for measuring caspase activity, comprising a hormone binding domain linked to a β-glucuronidase enzyme by a peptide comprising a caspase cleavage site, wherein the β-glucuronidase is inactive due to linkage to the hormone binding domain and release of the β-glucuronidase through caspase cleavage of the cleavage site restores activity of the β-glucuronidase.

10. A method for determining the presence or activity of a pre-determined protease in a biological sample, which comprises:

a) providing a chimeric protease detector protein comprising:

i) a repressor domain which represses activity of a normally biologically active protein fused thereto;

ii) a reporter domain comprising a protein having a detectable biological activity when not fused to the repressor domain; and

iii) a protease cleavage domain linking the repressor domain to the reporter domain, the protease cleavage domain comprising a structure that is cleaved by activity of the pre-determined protease;

b) adding the protease detector protein to the biological sample suspected of containing the pre-determined protease; and

c) measuring the detectable biological activity, if any, of the reporter domain, the occurrence and amount of the detectable biological activity being proportional to the occurrence and amount of the pre-determined protease in the biological sample.

11. The method of claim 10, wherein the biological sample comprises a biological fluid, tissue or cell extract and the protease detector protein is provided as an isolated protein.

12. The method of claim 10, wherein the biological sample comprises

intact cells in which the pre-determined protease, if present, is contained, and the protease detector protein is provided by introducing into the cells an expressible DNA construct that encodes the protein, under conditions whereby the protein is expressed.

13. The method of claim 12, wherein the expressible DNA construct is introduced into the cells by transient transformation.

14. The method of claim 12, wherein the expressible DNA construct is introduced into the cells by stable transformation.

15. The method of claim 10, adapted for determining the presence or amount of a plurality of pre-determined proteases.

16. The method of claim 15, wherein the plurality of proteases are detected by adding a plurality of protease detector proteins, each having a protease cleavage domain specifically cleaved by one of the pre-determined proteases, and each having a differentially detectable reporter domain.

17. The method of claim 15, wherein the plurality of proteases are detected by adding one or more modified protease detector proteins, each comprising a repressor domain linked to two different protease cleavage domain, each protease cleavage domain being linked to a differentially detectable reporter domain.

18. A method for determining if a test compound affects the amount or activity of a pre-determined protease, the method comprising:

a) providing a chimeric protease detector protein comprising:

i) a repressor domain which represses activity of a normally biologically active protein fused thereto;

ii) a reporter domain comprising a protein having a detectable biological activity when not fused to the repressor domain; and

iii) a protease cleavage domain linking the repressor

domain to the reporter domain, the protease cleavage domain comprising a structure that is cleaved by activity of the pre-determined protease;

b) preparing a test sample and a control sample, the test sample containing the pre-determined protease, the protease detector protein and the test compound, the control sample containing the pre-determined protease and the protease detector protein;

c) measuring the detectable biological activity, if any, of the reporter domain, in the test sample and the control sample; and

d) comparing the amount of the detectable biological activity in the test sample with that in the control sample, an increase or decrease of the activity in the test sample being indicative of the ability of the test compound to affect the amount or activity of the protease.

19. A test kit for detecting the presence or activity of a pre-determined protease, which comprises a container containing:

a) a chimeric protease detector protein comprising:

i) a repressor domain which represses activity of a normally biologically active protein fused thereto;

ii) a reporter domain comprising a protein having a detectable biological activity when not fused to the repressor domain; and

iii) a protease cleavage domain linking the repressor domain to the reporter domain, the protease cleavage domain comprising a structure that is cleaved by activity of the pre-determined protease;

b) optionally, at least one other reagent for using the protease detector protein; and

c) optionally, instructions for using the protease detector protein.

20. The test kit of claim 19, adapted for detection of a plurality of pre-determined proteases, which comprises a plurality of chimeric protease detector proteins.

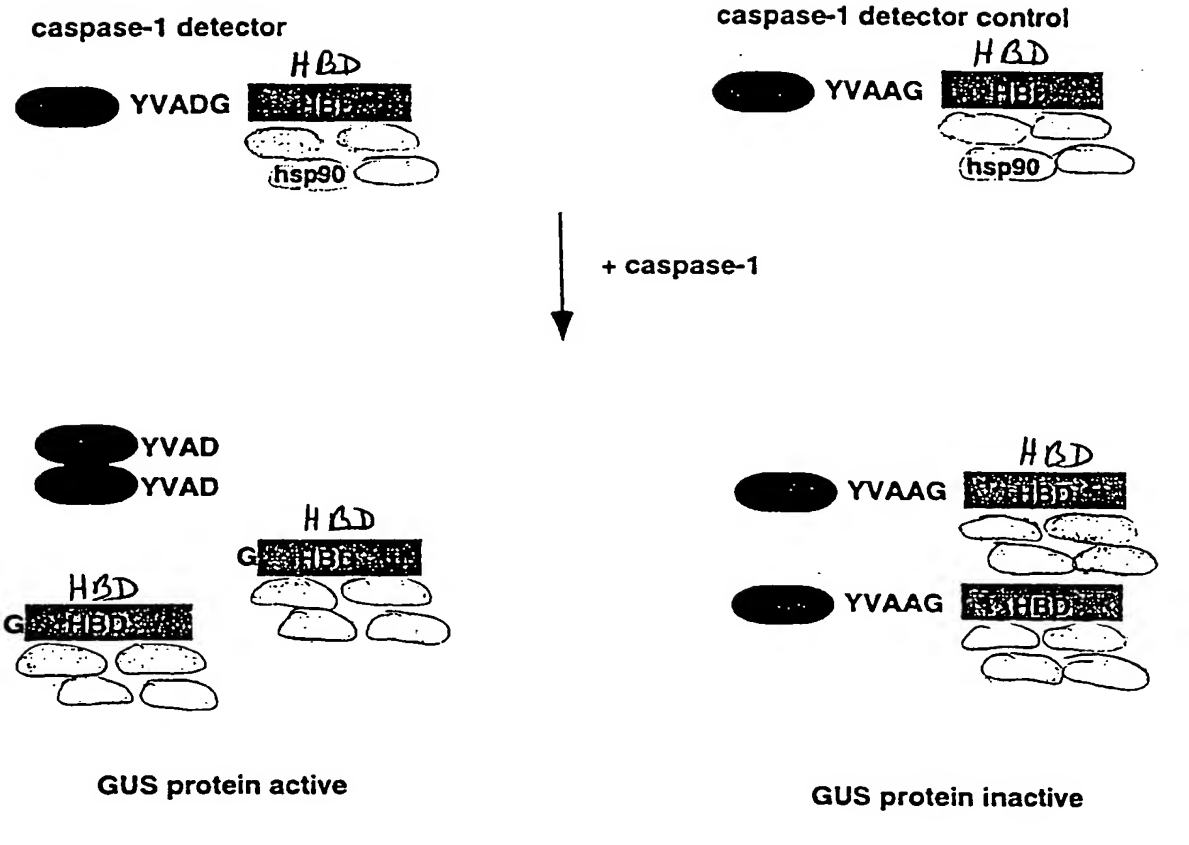
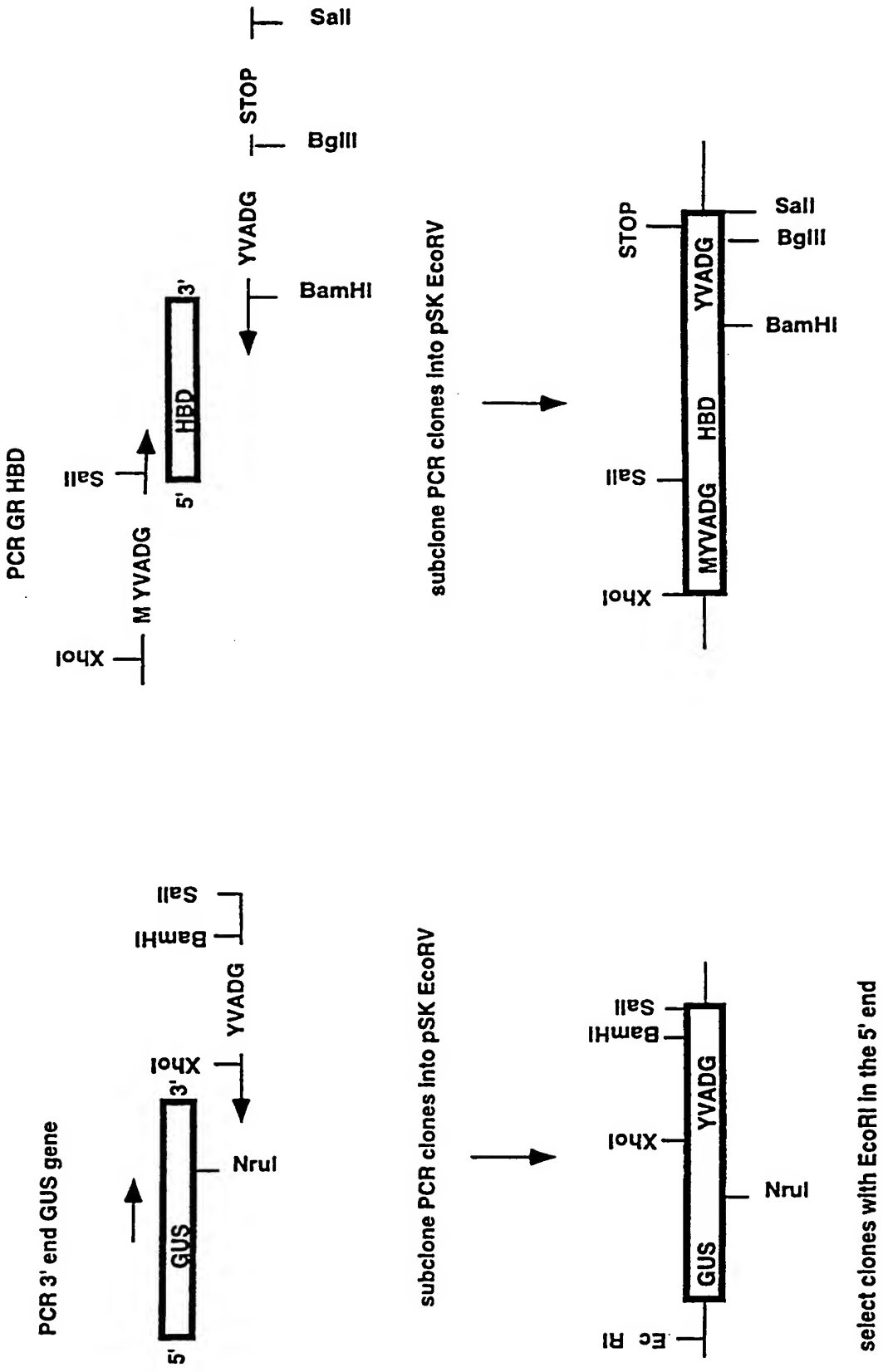

caspase-1 detector
YVADG
HBD
hsp90
caspase-1 detector control
YVAAG
HBD
hsp90
+ caspase-1
YVAD
YVAD
HBD
G
HBD
HBD
G
HBD
YVAAG
HBD
YVAAG
HBD
GUS protein active
GUS protein inactive

Fig.1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XhoI
M YVADG
SalI
HBD
5'
3'
YVADG
BamHI
STOP
BglII
SalI
subclone PCR clones into pSK EcoRV
XhoI
MYVADG
HBD
YVADG
SalI
BglII
BamHI
STOP
PCR 3' end GUS gene
5'
GUS
3'
XhoI
YVADG
BamHI
SalI
NruI
subclone PCR clones into pSK EcoRV
EcoRI
GUS
YVADG
XhoI
BamHI
SalI
NruI
select clones with EcoRI in the 5' end

Fig.2a

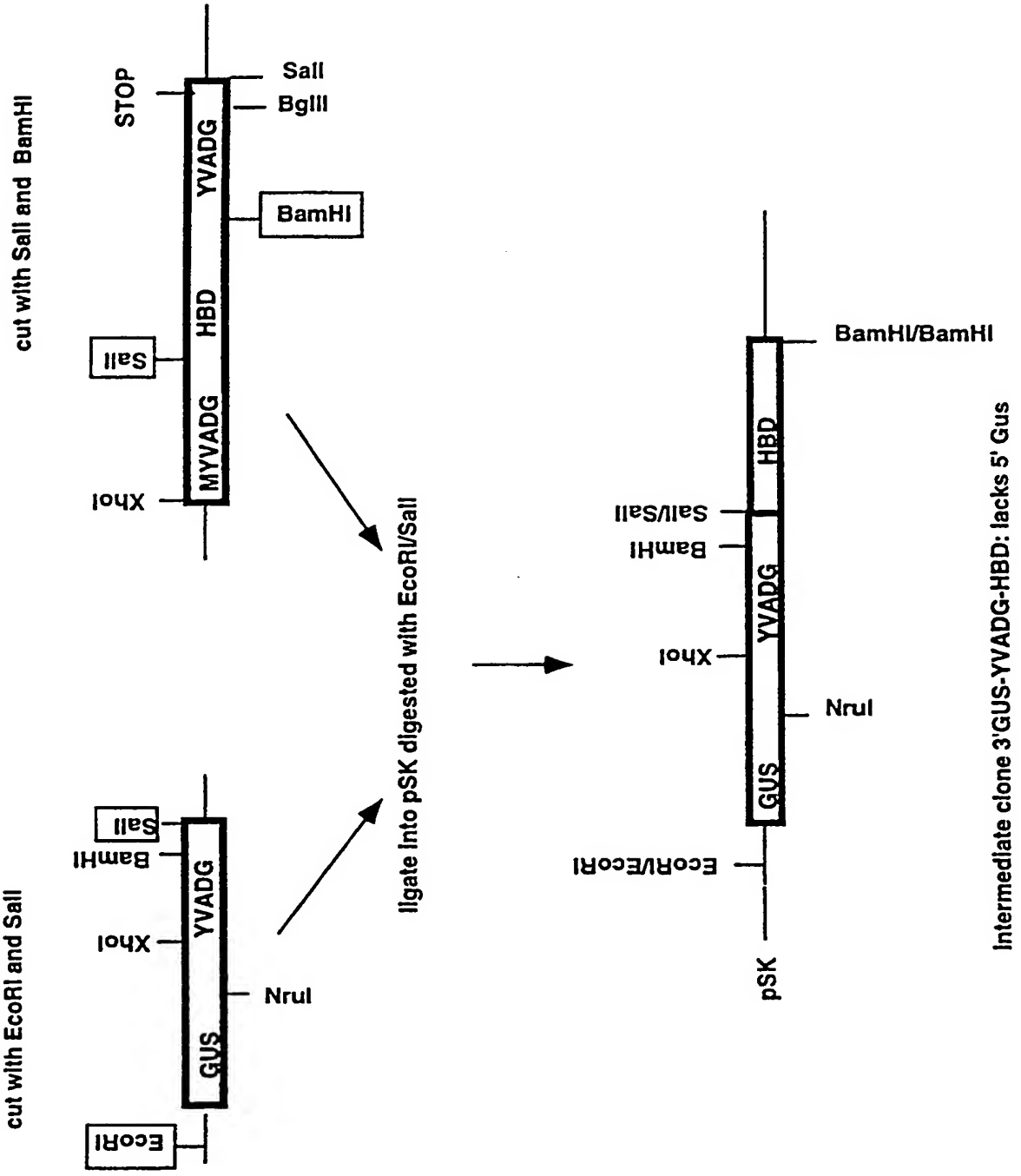

cut with SalI and BamHI
STOP
SalI
BglII
YVADG
BamHI
HBD
SalI
MYVADG
XhoI
cut with EcoRI and SalI
SalI
BamHI
XhoI
YVADG
NruI
GUS
EcoRI
ligate into pSK digested with EcoRI/SalI
BamHI/BamHI
HBD
SalI/SalI
BamHI
XhoI
YVADG
NruI
GUS
EcoRI/EcoRI
pSK
Intermediate clone 3'GUS-YVADG-HBD: lacks 5' Gus

Fig.2b

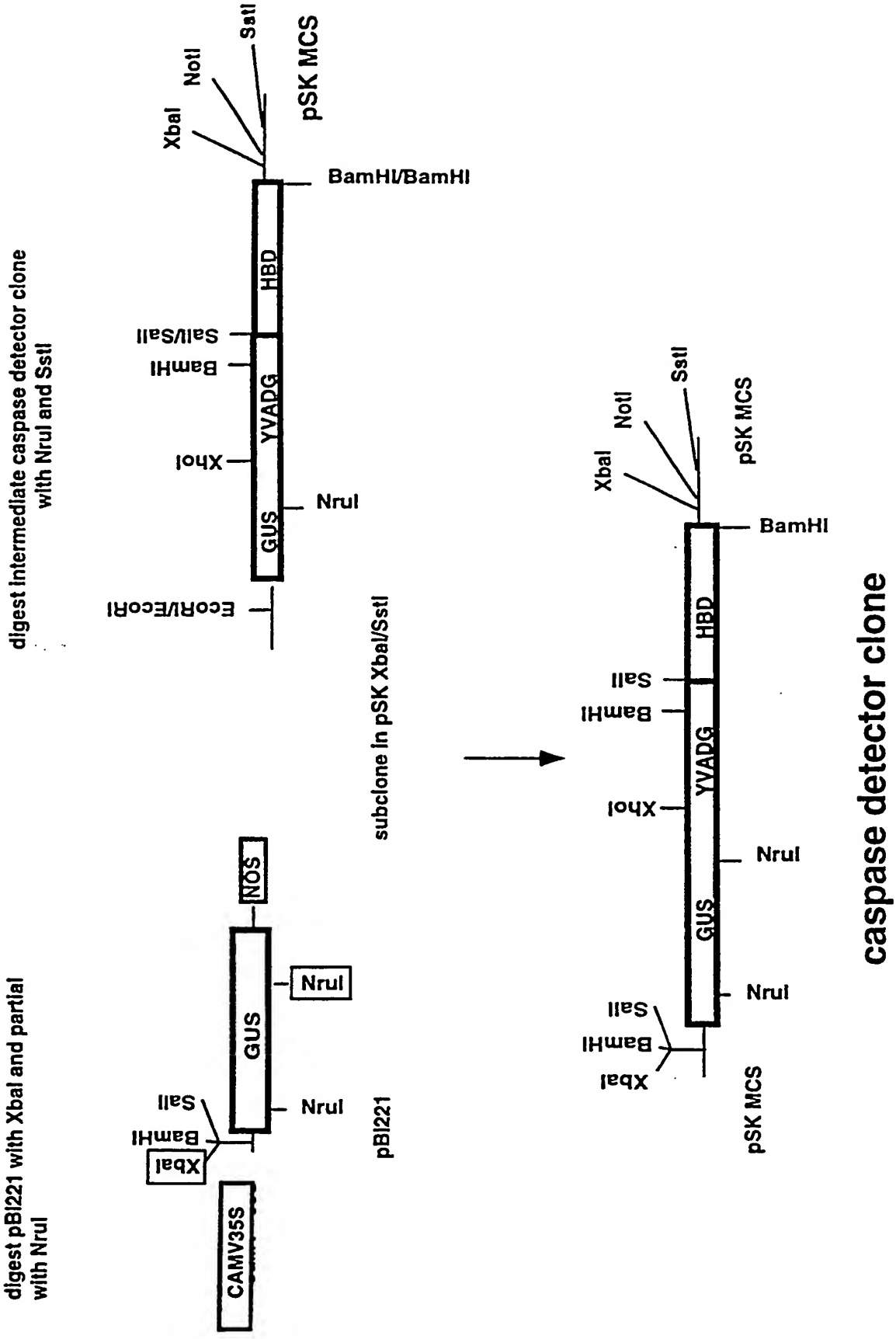

digest pBI221 with XbaI and partial with NruI
CAMV35S
XbaI
BamHI
SalI
NruI
GUS
NruI
NOS
pBI221
subclone in pSK XbaI/SstI
digest intermediate caspase detector clone with NruI and SstI
EcoRI/EcoRI
NruI
GUS
XhoI
BamHI
SalI/SalI
HBD
BamHI/BamHI
XbaI
NotI
SstI
pSK MCS
XbaI
BamHI
SalI
NruI
GUS
XhoI
NruI
BamHI
SalI
HBD
BamHI
XbaI
NotI
SstI
pSK MCS
pSK MCS
caspase detector clone

Fig.2c

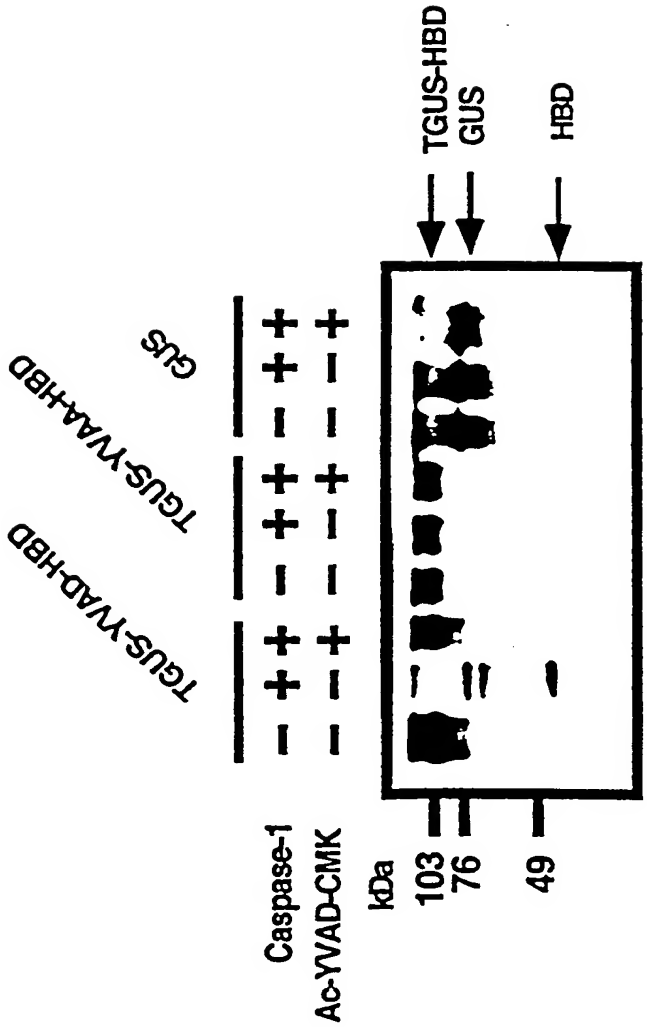
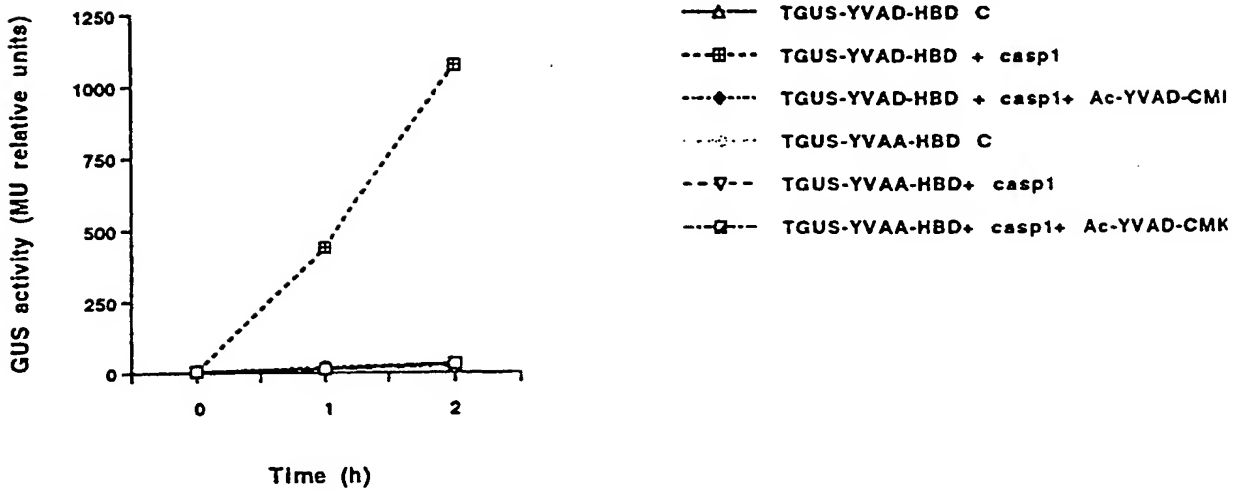

TGUS-HBD
GUS
HBD
GUS
TGUS-YVAA-HBD
TGUS-YVAD-HBD
Caspase-1
Ac-YVAD-CMK
kDa
103
76
49

Fig.3

A)


GUS activity (MU relative units)
1250
1000
750
500
250
0
0
1
2
Time (h)
Ret
Ret+casp1
Ret+ casp1+Ac-YVAD-CMK
TGUS-YVAD-HBD C
TGUS-YVAD-HBD + casp1
TGUS-YVAD-HBD + casp1+ Ac-YVAD-CMI
TGUS-YVAA-HBD C
TGUS-YVAA-HBD+ casp1
TGUS-YVAA-HBD+ casp1+ Ac-YVAD-CMK

B)

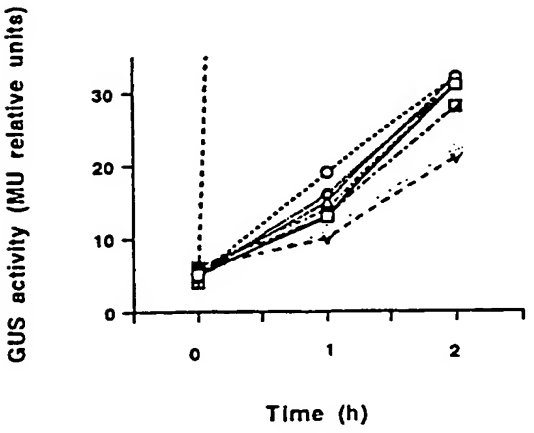

GUS activity (MU relative units)
30
20
10
0
0
1
2
Time (h)

Fig.4

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<110> Lam, Eric
Del Pozo, Olga

<120> Compositions and Methods for Detection of Active Proteases

<130> RutC 99-0034 (13216/00024)

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/11893

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07K 1/00, 14/00, 17/00
US CL :530/350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

INDEX BIOSCIENCE, BIOSIS, CAPLUS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	EVANS et al. Expression and characterization of chimeric rDNA proteins engineered for purification and enzymatic cleavage. Protein Expr. Purif. 15 April 1991, Vol. 2, pages 205-213, especially pages 205-210.	1-9 -- 1-9
X -- Y	FIELDS et al. The two-hybrid system: an assay for protein-protein interactions. Trends Genet. 01 August 1994, Vol. 10, No. 8, pages 286-292, especially pages 286-291.	1-9 -- 1-9
X -- Y	LIEBIG et al. Proteinase trapping: Screening for viral proteinase mutants by α complementation. Proc. Natl. Acad. Sci. USA. 15 July 1991, Vol. 88, pages 5979-5983, see entire document.	1-20 -- 1-20

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 15 AUGUST 2000	Date of mailing of the international search report 31 AUG 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MANJUNATH RAO Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/11893

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 5,861,161 A (COHEN ET AL.) 19 January 1999 (19-01-99), see entire document.	1-20 -- 1-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06070

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 1/13, 1/20, 5/00, 5/02, 5/08; C12Q 1/00
US CL :435/4, 252.3, 254.11, 325, 366; 530/350; 536/23.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 252.3, 254.11, 325, 366; 530/350; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,691,183 A (FRANZUSOFF et al.) 25 November 1997, see entire document.	1-56
Y	US 5,716,622 A (DARNELL, Jr. et al.) 10 February 1998, see entire document.	1-56
Y	SARUBBI et al. A high throughput assay for inhibitors of HIV-1 protease, screening of microbial metabolites. FEBS Letters. February 1991, Vol. 279, No. 2, pages 265-269, see entire document.	1-56
Y	WEARNE, S.J. Factor Xa cleavage of fusion proteins, elimination of non-specific cleavage by reversible acylation. FEBS Letters. April 1990, Vol. 263, No. 1, pages 23-26, see entire document.	1-56

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
10 JUNE 1999	07 JUL 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer IREM YUCEL Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

Intemational application No.
PCT/US99/06070

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, STN (CAPLUS), DIALOG (MEDLINE, BIOSIS, SCISEARCH, PASCAL)

TERMS: Inventor's names, fusion?, chim?, protein?, polypeptide?, linker or spacer, cleav? site, protease?, peptidase? proteinase? screen?, test?, assay?, activit?, function?, reporter?, repress?, regulat?, caspase?, ICE, NEDD, CED